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## BISPECIFIC CD3 × CD19 DIABODY FOR T CELL-MEDIATED LYSIS OF MALIGNANT HUMAN B CELLS

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For the treatment of minimal residual disease in patients with leukemias and malignant lymphomas, we constructed a heterodimeric diabody specific for human CD19 on B cells and CD3ε chain of the T cell receptor complex. The bispecific diabody was expressed in *Escherichia coli* using a vector containing a dicistronic operon for co-secretion of V<sub>H</sub>3-V<sub>L</sub>19 and V<sub>H</sub>19-V<sub>L</sub>3 single-chain Fv fragments (scFv). It was purified in one step by immobilized metal affinity chromatography (IMAC) from the periplasmic extract and culture medium. Flow cytometry experiments revealed specific interactions of the diabody with both CD3 and CD19 positive cells, to which it bound with affinities close to those of the parental scFvs. It was less stable than anti-CD3 scFv but more stable than anti-CD19 scFv when incubated in human serum at 37°C. In cytotoxicity tests, the diabody proved to be a potent agent for retargeting peripheral blood lymphocytes to lyse tumor cells expressing the CD19 antigen. The efficiency of cell lysis compared favorably with that obtained with a bispecific antibody (BsAb) of the same dual specificity that was prepared by the quadroma technique. *Int. J. Cancer* 77:763–772, 1998.

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B cell leukemias and malignant lymphomas represent a heterogeneous group of hematological malignancies occurring in blood, lymph nodes and bone marrow, which frequently disseminate throughout the body. The most common forms of non-Hodgkin's lymphoma (NHL) are derived from the B cell lineage. The incidence of NHL (6–17/100,000) continues to increase worldwide at about 4% a year. Although NHL can be treated with reasonable success at early and intermediate stages, the results of conventional chemotherapy and radiation in advanced stages remain disappointing. This holds particularly true for the prevalent low-grade lymphomas. A fairly large number of patients relapse, and most remissions cannot be extended beyond minimal residual disease. In this case, high-dose chemotherapy combined with total body irradiation together with the support of an autologous bone marrow transplantation (BMT) or peripheral blood stem cell transplantation (PBSCT) offers an alternative means of curing the disease. However, an important issue still to be solved concerns the presence of residual tumor cells in the patient that give rise to a recurrence of the leukemia or lymphoma.

To eradicate residual tumor cells, BsAbs have been proposed as a means of recruiting cytotoxic T cells for killing tumor cells (Fanger *et al.*, 1992). For example, clinical studies have shown tumor regression in patients treated with BsAb directed against tumor antigens and the CD3 component of the T cell receptor complex, respectively (Canevari *et al.*, 1995; Nitta *et al.*, 1990). One of the best targets for bispecific antibodies on malignant human B cells is CD19 (Grossbard *et al.*, 1992). This antigen is expressed on virtually all B-lineage malignancies from acute lymphoblastic leukemia (ALL) to NHL. Moreover, it is not shed and is absent from hemopoietic stem cells, plasma cells, T cells and other tissues. A potential disadvantage is that normal B cells may also be killed by CD3 × CD19 BsAb treatment. However, these cells are rapidly replaced by differentiation from the stem cell pool.

Various strategies have been utilized for the creation of BsAbs. Heteroconjugates have been produced by chemical cross-linking of 2 monoclonal antibodies (MAb) (Anderson *et al.*, 1992) or Fab' fragments (Brennan *et al.*, 1985). Alternatively, BsAbs were pro-

duced using hybrid hybridoma (quadroma) technology (Bohlen *et al.*, 1993; Csoka *et al.*, 1996). A major limitation of this procedure is the production of inactive antibodies due to the random L-H and H-H associations. Only about 15% of the antibody produced by the quadroma is of the desired specificity (Milstein and Cuello, 1983). The correct BsAb must then be purified in a costly procedure from a large quantity of other very similar molecules. A further limitation of the quadroma BsAb from rodent cell lines is their immunogenicity. Repeated doses of rodent antibodies elicit an anti-immunoglobulin response, referred to as HAMA (human anti-murine antibody).

Some of the limitations of MAbs as therapeutic agents have recently been addressed by genetic engineering (Winter and Milstein, 1991) including a few methods for BsAb fragment production (Carter *et al.*, 1995). Bispecific F(ab')<sub>2</sub> have been created either by chemical coupling from Fab' fragments expressed in *E. coli* (Shalaby *et al.*, 1992) or by heterodimerization through leucine zippers (Kostelny *et al.*, 1992). Even smaller BsAb fragments have been constructed based on scFv: the association of V<sub>H</sub> and V<sub>L</sub> domains is stabilized by a flexible polypeptide linker (Bird *et al.*, 1988). The genetic engineering of 2 scFvs linked with a third polypeptide linker, as initially suggested by Huston *et al.*, (1991), has now been carried out in several laboratories for the production of bispecific single-chain antibody segments (scFv)<sub>2</sub> with a potential anti-tumor activity (Gruber *et al.*, 1994; Mack *et al.*, 1995).

An alternative BsAb fragment is the scFv heterodimer diabody (Holliger *et al.*, 1993). It is formed by the non-covalent association of 2 single-chain fusion products consisting of the V<sub>H</sub> domain from one antibody connected by a short linker to the V<sub>L</sub> domain of another antibody (Atwell *et al.*, 1996; Holliger *et al.*, 1996; Zhu *et al.*, 1996). The 2 antigen binding domains have been shown by crystallographic analysis to be on opposite sides of the complex such that they are able to cross-link 2 cells (Perisic *et al.*, 1994).

Starting with the mRNA of hybridoma cells HD37 (Pezzutto *et al.*, 1987) and OKT3 (Kung *et al.*, 1979), we previously constructed recombinant scFv antibody fragments specific for the human B cell antigen CD19 (Kipriyanov *et al.*, 1996) and the CD3ε chain of the human CD3/T cell receptor complex (Kipriyanov *et al.*, 1997b). Here, we describe the construction and production of a bispecific diabody in bacteria with dual specificity for both the human B cell antigen CD19 and CD3/TCR. The diabody has been compared with parental scFvs with respect to its stability in human serum at 37°C and binding affinity to both CD19-positive human B cells and CD3-positive human T cells. In cytotoxic assays, the CD3 × CD19 diabody was able to retarget human PBL to malignant B cells. The efficiency of cell lysis compared favorably with that obtained with a BsAb of the same dual specificity prepared by the quadroma technique.

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## MATERIAL AND METHODS

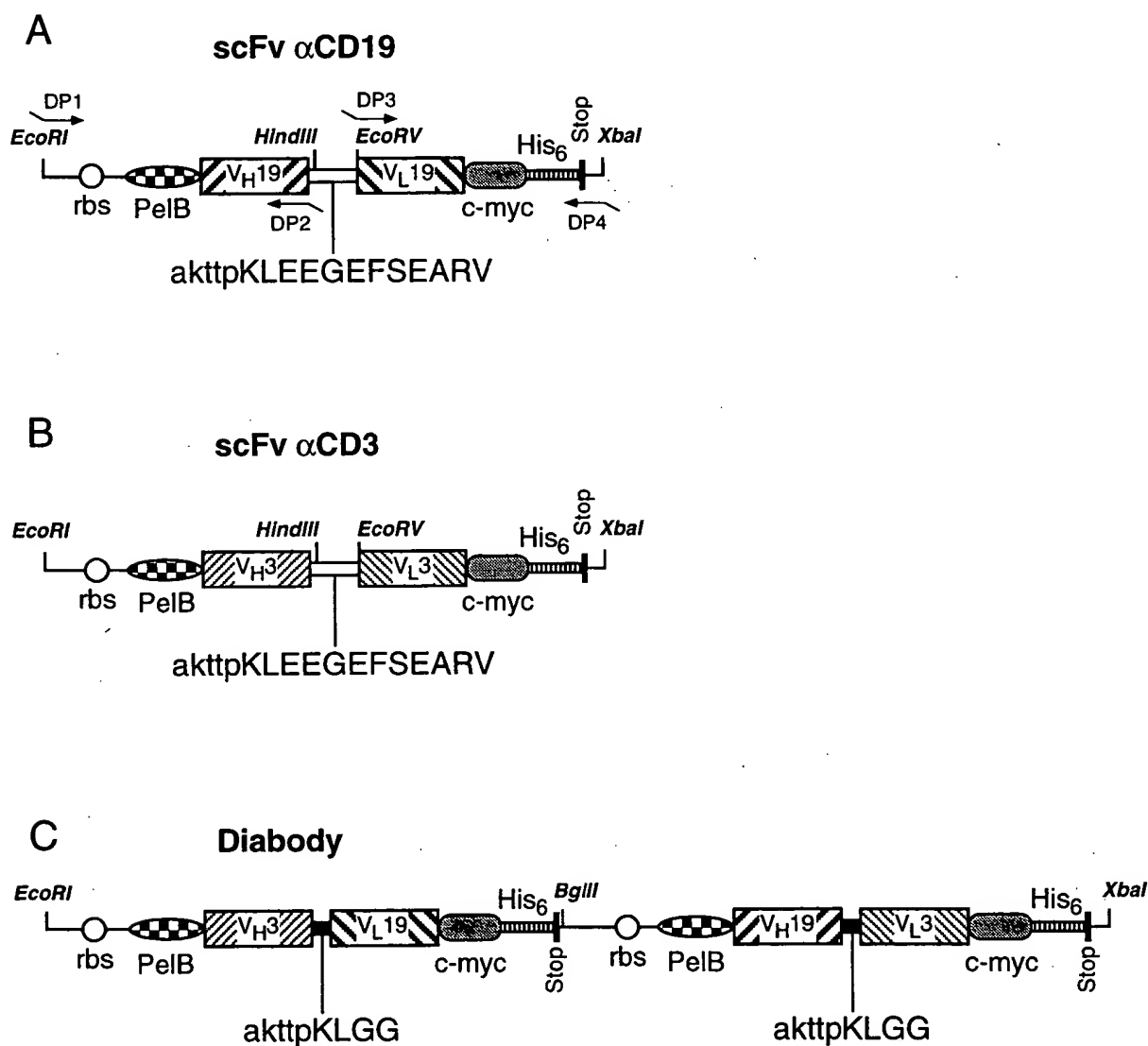
## Monoclonal antibodies

The CD3  $\epsilon$ -chain-specific hybridoma cell line OKT3 secreting an IgG<sub>2a</sub> MAb (Kung *et al.*, 1979) was obtained from the ATCC (Rockville, MD). The HD37 cell line produces a MAb (IgG<sub>1</sub>) reactive with the human CD19 molecule and has been described in detail (Pezzutto *et al.*, 1987). Monoclonal antibodies were produced in a miniPERM bioreactor (Heraeus, Osterode, Germany) and purified by affinity chromatography on a Protein-A Sepharose CL-4B column (Pharmacia, Uppsala, Sweden). Isolation and characterization of hybridoma OKT3  $\times$  HD37 has been described (Cs6ka *et al.*, 1996).

## Vector construction

The *E. coli* K12 strain XL1-Blue (Stratagene, La Jolla, CA) was used as the cloning and expression host. Plasmids pHOG- $\alpha$ CD19 and pHOG-dmOKT3 encoding the scFv fragments derived from

hybridoma HD37 specific for human CD19 (Kipriyanov *et al.*, 1996) and OKT3 specific for human CD3 (Kipriyanov *et al.*, 1997b), respectively, were used for assembly of the diabody to create the expression plasmid pKID3  $\times$  19. Briefly, a PCR fragment of the V<sub>H</sub> domain of anti-CD19 preceded by a *Bgl*II site and followed by a segment coding for a LysLeuGlyGly linker was generated using the primers DP1, 5'-TCACACAGAATTCTT AGATCTATTAAAGAGGAGAAATTAACC and DP2, 5'-AGCACGATATCACC GCCAAGCTTGGGTGTTGTTTGGC (Fig. 1). The PCR fragment was digested with *Eco*RI and *Eco*RV and ligated with the *Eco*RI/*Eco*RV linearized plasmid pHOG-dmOKT3, generating the vector pHOG19-3. The PCR fragment of the V<sub>L</sub> domain of anti-CD19 followed by a segment coding for a c-myc epitope and a hexahistidyl tail was generated using the primers DP3, 5'-AGCACACAAGCTTGGCGGTGATATCTTGCTCACCCAAA-CTCCA plus DP4, 5'-AGCACACTCTAGAGACACACAGATCT-TTAGTGATGGTGATGGTGATGTGAGTTTAGG. The PCR frag-



**FIGURE 1** – Schematic representation of operons encoding anti-CD19 and anti-CD3 scFvs in plasmid pHOG21 (Kipriyanov *et al.*, 1997a) (a and b, respectively) and dicistronic operon encoding the bispecific anti-human CD3  $\times$  CD19 diabody in plasmid pKID3  $\times$  19 (c). The positions of primers and most important restriction sites used for constructing the plasmids are shown. The locations of ribosome binding sites (rbs), pelB leader sequences (pelB), c-myc epitopes (c-myc), hexahistidyl tags (His<sub>6</sub>) and stop codons (Stop) are indicated. Amino acid sequences of linkers connecting V-domains are shown below each drawing. In the linker, amino acids derived from the C<sub>H</sub>1 domain are indicated by small letters and residues introduced artificially are shown in block letters.

ment of the V<sub>L</sub> domain of anti-CD19 contained a BglII site near the 3' end of the coding strand. It was digested with HindIII and XbaI and ligated with the HindIII/XbaI linearized plasmid pHOG-dmOKT3, generating the vectors pHOG3-19. The expression plasmid pKID3 × 19 for cosecretion of the two hybrid scFvs was constructed by ligation of the BglII/XbaI restriction fragment from pHOG3-19 comprising the vector backbone and the BglII/XbaI fragment from pHOG19-3. All sequences encoding hybrid scFv fragments were verified by the dideoxynucleotide method (Sanger *et al.*, 1977).

#### ScFv and diabody expression and purification

Bacterial growth, induction and isolation of periplasmic extracts was performed as previously described (Kipriyanov *et al.*, 1996, 1997a, b). For isolation of anti-CD3 and anti-CD19 scFv, the culture supernatant and the soluble periplasmic extract were combined and concentrated using Amicon YM10 membranes with a 10 kDa cut-off (Amicon, Witten, Germany) followed by thorough dialysis against 50 mM Tris-HCl, 1 M NaCl, pH 7.0. The diabody was concentrated by ammonium sulfate precipitation (final concentration 70% of saturation) as recommended (Atwell *et al.*, 1996). The protein precipitate was collected by centrifugation (30,000g, 4°C, 30 min) and dissolved in 1/10 of the initial volume of 50 mM Tris-HCl, 1 M NaCl, pH 7.0. Purification was achieved by IMAC as previously described (Kipriyanov *et al.*, 1997a). The final purification of scFv-αCD3 (I<sub>a</sub> = 7.52) and scFv-αCD19 (I<sub>a</sub> = 6.19) was achieved by ion-exchange chromatography on a MonoS HR5/5 column (Pharmacia) in 50 mM MES, pH 6.0, or on a MonoQ HR5/5 column (Pharmacia) in 20 mM Tris-HCl, pH 8.0, respectively, with a linear 0–1 M NaCl gradient. The purified antibody preparations were dialyzed against PBS (15 mM Na-phosphate, 0.15 M NaCl, pH 7.0). All purification procedures were performed at 4°C. For long-time storage, diabody and scFv were frozen in the presence of BSA (final concentration 10 mg/ml) or 10% FCS and stored at –80°C.

#### Measurement of protein concentration

Protein concentrations were determined by the Bradford (1976) dye-binding assay, using the Bio-Rad (Munich, Germany) protein assay kit. The concentrations of purified diabody, scFv-αCD3 and scFv-αCD19 were determined from the A<sub>280</sub> values using the extinction coefficients ε<sup>1mg/ml</sup> = 1.89, 1.84 and 1.82, respectively, calculated according to Gill and von Hippel (1989).

#### SDS-PAGE and size-exclusion chromatography

SDS-PAGE was performed according to Laemmli (1970) under reducing conditions. Western blot analyses using either rabbit serum A (Breitling *et al.*, 1991) recognizing the N-terminus of the processed antibody fragment (without a pelB leader) or mouse MAb 9E10 (IC Chemikalien, Ismaning, Germany) specific for a peptide of the c-myc oncoprotein were performed as previously described (Kipriyanov *et al.*, 1994). Analytical gel filtration of the diabody and scFv preparations was performed in PBS using a Superdex 75 HR10/30 column (Pharmacia). Sample volume and flow rate were 200 µl and 0.5 ml/min, respectively. The column was calibrated with a low molecular weight gel filtration calibration kit (Pharmacia).

#### Flow cytometry

The human CD3<sup>+</sup>/CD19<sup>–</sup> acute T cell leukemia line Jurkat and the CD19<sup>+</sup>/CD3<sup>–</sup> B cell line JOK-1 were used for flow cytometry, performed as previously described (Kipriyanov *et al.*, 1996). In brief, 5 × 10<sup>5</sup> cells in 50 µl RPMI 1640 medium (GIBCO BRL, Eggenstein, Germany) supplemented with 10% FCS and 0.1% sodium azide (referred to as complete medium) were incubated with 100 µl of a recombinant antibody preparation for 45 min on ice. After washing with complete medium, the cells were incubated with 100 µl of 10 µg/ml anti c-myc MAb 9E10 (IC Chemikalien) in the same buffer for 45 min on ice. After a second washing cycle, the cells were incubated with 100 µl of FITC-labeled goat anti-mouse IgG (GIBCO BRL) under the same conditions as before. The cells

were then washed again and resuspended in 100 µl of 1 µg/ml solution of propidium iodide (Sigma, Deisenhofen, Germany) in complete medium to exclude dead cells. The relative fluorescence of stained cells was measured using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

#### Affinity determination

Affinities of MAb HD37 (Pezzutto *et al.*, 1987) and scFv-αCD19 (Kipriyanov *et al.*, 1996) were determined by cellular RIA. Antibody (100 µg) was labeled with 1 mCi [<sup>125</sup>I]-iodide by the chloramine T method (Greenwood *et al.*, 1963). RIA was performed in a flexible polyvinyl chloride microtiter plate blocked with PBS containing 0.2% (w/v) gelatin at 4°C overnight. After washing, 10<sup>6</sup> JOK-1 cells in 50 µl PBS containing 0.2% gelatin and 5% (v/v) pooled human IgG (Venimmun Behringwerke, Marburg, Germany) were incubated in triplicate with increasing amounts of the radiolabeled antibody preparation for 1 hr at room temperature. The plate was washed and aspirated 3 times using PBS/0.2% gelatin. The dried plate was sliced, and the radioactivity in individual wells was measured using a gamma-counter. Affinity constants were determined by a Scatchard (1949) plot analysis.

Apparent affinities of diabody and scFv were determined from competitive inhibition assays as previously described (Kipriyanov *et al.*, 1997b). In brief, increasing concentrations of purified antibody fragment were added to a subsaturating concentration of FITC-labeled MAb OKT3 or HD37 and incubated with Jurkat or JOK-1 cells, respectively, as described above for FACScan analysis. Fluorescence intensities of stained cells were measured as described above. Binding affinities were calculated according to the following equation derived from that of Schodin and Kranz (1993):

$$K_{a(I)} = (1 + [\text{FITC-MAB}] \times K_{a(\text{MAB})}) / \text{IC}_{50}$$

where I is the unlabeled inhibitor (diabody or scFv), [FITC-MAB] is the concentration of FITC-labeled MAb, K<sub>a(MAB)</sub> is the binding affinity of MAb and IC<sub>50</sub> is the concentration of inhibitor that yields 50% inhibition of binding. Affinity constant values of 1.2 × 10<sup>9</sup> M<sup>–1</sup> and 2.5 × 10<sup>9</sup> M<sup>–1</sup> were taken for MAb OKT3 (Adair *et al.*, 1994) and HD37 (determined by RIA), respectively.

#### Analyses of diabody and scFv stability

The antibody fragments were stored in freshly prepared human serum from a healthy donor at 37°C at a concentration 20 µg/ml. At given time points, 250 µl aliquots were taken under sterile conditions, frozen and kept at –80°C. Activities of samples after storage were determined by flow cytometry.

#### Preparation and stimulation of effector cells

Human PBMCs were isolated from the buffy coat of healthy donors by Ficoll/Hypaque (Pharmacia) density gradient centrifugation. The PBMC interphase was washed twice in PBS and used immediately as effector cells. Cultures of PBMC were grown using RPMI 1640 (GIBCO BRL) supplemented with 2% heat inactivated FCS (GIBCO BRL), 2 mM glutamine, 1 mM pyruvate and 0.05 mM 2-ME. To obtain CTLs, PBMCs were cultured at a concentration of 2 × 10<sup>6</sup> ml in medium containing anti-CD3 MAb OKT3 (5 µg/ml) and recombinant human IL-2 (20 U/ml) (EuroCetus, Amsterdam, The Netherlands). After 4 days, the cells were washed twice to remove remaining antibody and cultured overnight in medium alone. The cytotoxicity assay was performed on day 5.

#### Cytotoxicity assay

The CD19-expressing Burkitt's lymphoma cell lines Raji and Namalwa were used as target cells. Cells were cultured in RPMI 1640 (GIBCO BRL) supplemented with 10% heat inactivated FCS (GIBCO BRL), 2 mM glutamine and 1 mM pyruvate at 37°C in a humidified atmosphere containing 7.5% CO<sub>2</sub>. The cytotoxic T cell assays were carried out in RPMI 1640 medium supplemented with 10% FCS, 10 mM HEPES, 2 mM glutamine, 1 mM pyruvate and 0.05 mM 2-ME. Cytotoxic activity was assessed using a standard

[<sup>51</sup>Cr]-release assay;  $2 \times 10^6$  target cells were labeled with 200  $\mu$ Ci Na<sup>51</sup>CrO<sub>4</sub> (Amersham-Buchler, Braunschweig, Germany) followed by 4 washing cycles and resuspended in medium at a concentration  $2 \times 10^5$ /ml. Effector cells were adjusted to a concentration of  $5 \times 10^6$  ml. Increasing amounts of CTLs in 100  $\mu$ l were titrated to  $10^4$  target cells/well in 50  $\mu$ l. Antibodies (50  $\mu$ l) were added to each well. The whole assay was set up in triplicate and incubated for 4 hr at 37°C. Supernatant (100  $\mu$ l) was harvested and assayed for [<sup>51</sup>Cr] release in a gamma-counter (Cobra Auto Gamma; Canberra Packard, Dreieich, Germany). Maximum release was determined by incubating the target cells in 10% SDS, and spontaneous release was determined by incubating the cells in medium alone. Specific lysis (%) was calculated as: (experimental release - spontaneous release)/(maximal release - spontaneous release)  $\times$  100.

## RESULTS

### Diabody design and construction

Single-chain Fv fragments derived from the hybridomas HD37 (Pezzutto *et al.*, 1987) and OKT3 (Kung *et al.*, 1979) were used for creating a bispecific CD3  $\times$  CD19 diabody. A significant increase in the stability of the OKT3 anti-CD3 scFv was achieved by substituting a serine for cysteine in position 100A of the V<sub>H</sub> domain (Kipriyanov *et al.*, 1997b). The C-terminus of a V<sub>H</sub> domain was connected to the N-terminus of a V<sub>L</sub> domain of another specificity using a short rigid linker to restrict intra-chain pairing of V<sub>H</sub> and V<sub>L</sub>. The linker design was based on our strategy of cloning antibody V<sub>H</sub> domains using an anti-sense primer complementary to the 5'-region of the  $\gamma$  chain C<sub>H</sub>1 domain gene (Kipriyanov *et al.*, 1996, 1997b). Seven amino acids introduced by this primer formed a major part of the linker (Fig. 1). The plasmid pKID3  $\times$  19 was constructed to express the CD3  $\times$  CD19 diabody by co-secretion of the 2 hybrid scFvs V<sub>H</sub>3-V<sub>L</sub>19 and V<sub>H</sub>19-V<sub>L</sub>3 from a dicistronic operon (Fig. 1). The operon is under the transcriptional control of the wt lac promoter/operator, which is inducible with IPTG. Each hybrid scFv is preceded by a pelB leader sequence to direct secretion to the periplasmic space of *E. coli*. Both hybrid scFv genes are followed by nucleotide sequences coding for a c-myc tag for immunodetection and a hexahistidyl tail for purification of recombinant product using IMAC (Fig. 1).

### Diabody expression and purification

The CD3  $\times$  CD19 diabody was secreted from *E. coli* cells transformed with the pKID3  $\times$  19 plasmid. Relatively equal amounts of soluble hybrid scFv V<sub>H</sub>3-V<sub>L</sub>19 and V<sub>H</sub>19-V<sub>L</sub>3 were detected in crude periplasmic extracts by Western blot analysis using either MAb 9E10 specific to the C-terminal c-myc epitope or by serum A specific for the N-terminus of processed (without pelB leader) antibody fragment (data not shown). FACScan analyses of crude periplasmic extracts demonstrated that the diabody produced by bacteria specifically interacted with both CD3-positive Jurkat cells and CD19-positive JOK-1 cells. In contrast, neither hybrid scFv alone bound these cell lines (data not shown).

To obtain higher yields of soluble antibody fragments, we added 0.4 M sucrose to bacterial cells that had been induced with IPTG. We have shown that under these conditions the yield of soluble scFv can be increased up to 150-fold (Kipriyanov *et al.*, 1997a). The diabody was found to accumulate in the *E. coli* periplasm and was also released into the culture medium (data not shown). After concentrating by ammonium sulfate precipitation and purification by IMAC, yields of 2.0–2.5 mg/l with a purity greater than 95% were achieved (Fig. 2a). In contrast, ammonium sulfate was shown to be rather ineffective for precipitating monospecific anti-CD19 and anti-CD3 single chain Fv fragments that were isolated in parallel. Much higher yields of the purified scFv antibody fragments were obtained by concentrating by ultrafiltration prior to IMAC (data not shown).

### Analyses of diabody and scFv by SDS-PAGE and size-exclusion FPLC

Purified antibody fragments were analyzed by electrophoresis on 12% SDS polyacrylamide gels. Under these conditions, the diabody was resolved into 2 protein bands corresponding to the calculated M<sub>r</sub> of 28,900 for scFv V<sub>H</sub>19-V<sub>L</sub>3 and 29,300 for scFvs V<sub>H</sub>3-V<sub>L</sub>19 (Fig. 2a). Anti-CD3 and anti-CD19 scFvs appeared as single bands (calculated M<sub>r</sub> are 29,800 and 30,400, respectively).

An analysis of antibody fragments by gel-filtration on a Superdex 75 column is shown in Figure 2b. The scFv- $\alpha$ CD3 consisted only of monomers, the scFv- $\alpha$ CD19 of monomers, dimers and a multimeric form and the bispecific diabody only of dimers. The apparent m.w. of the diabody deduced from its chromatographic mobility was lower than its actual m.w., reflecting its compact structure (Perisic *et al.*, 1994).

### Antigen binding specificity and affinity of diabody and scFv

Flow cytometry experiments demonstrated a specific interaction of the diabody with both human CD19-positive JOK-1 and CD3-positive Jurkat cells. The fluorescence intensities were fairly comparable to those obtained using the parental monospecific scFvs at similar concentrations (Fig. 3).

The CD19 and CD3 binding affinities of the diabody were estimated by competitive binding to human JOK-1 and Jurkat cells in the presence of either FITC-labeled MAb HD37 (anti-CD19) or OKT3 (anti-CD3). The IC<sub>50</sub> value of diabody competing with FITC-OKT3 for binding T-cells was similar to that of scFv- $\alpha$ CD3 (Fig. 4b; Table I). In contrast, scFv anti-CD19 competed with FITC-HD37 more effectively than the bispecific diabody (Fig. 4a), probably because a significant amount of this scFv fragment was in a dimeric and multimeric form (Fig. 2b). Direct affinity measurements of radio-iodinated anti-CD19 MAb HD37 and scFv demonstrated that both have very similar affinity constants in the sub-nanomolar range (Table I), reflecting the bivalent nature of the scFv anti-CD19.

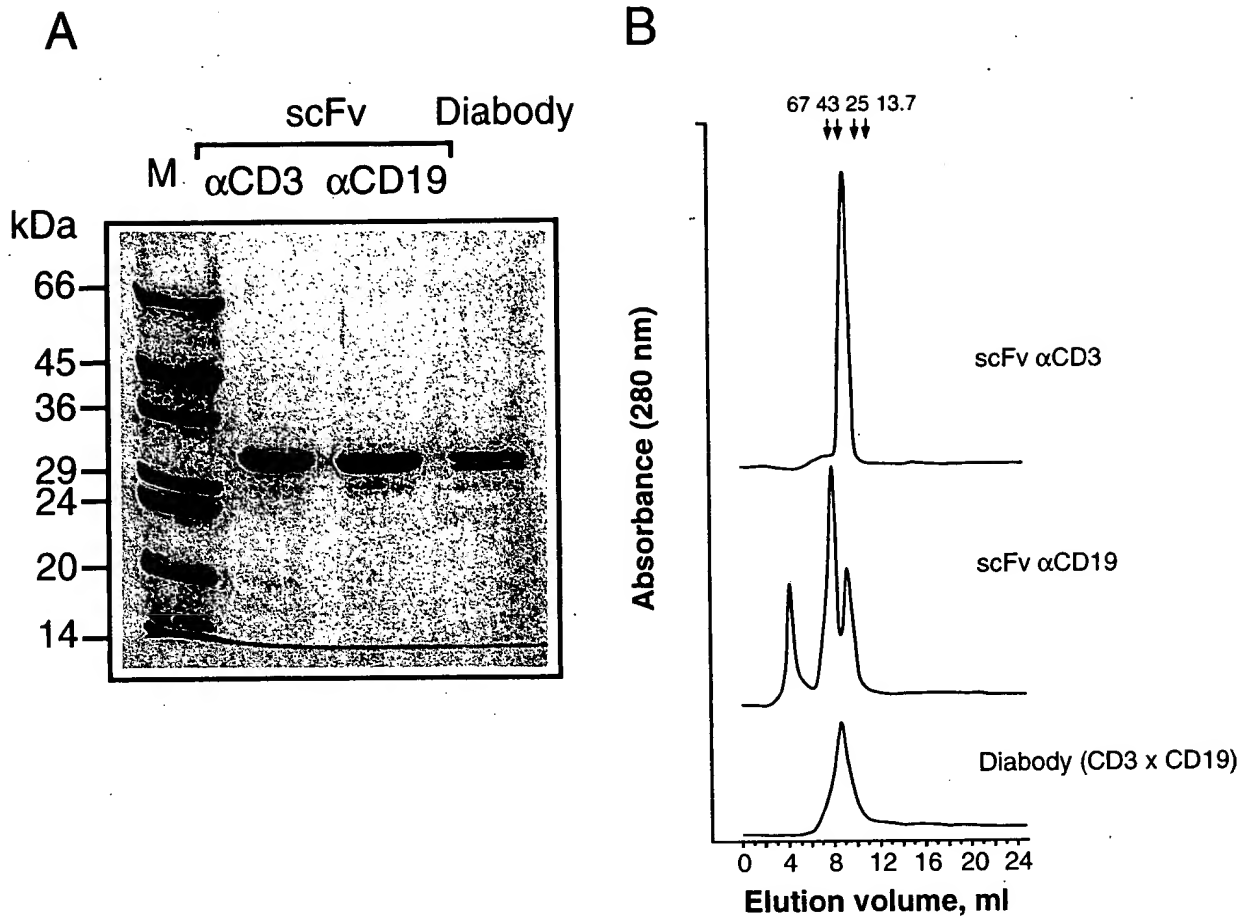
Table I summarizes the results of affinity measurements. The similarity in affinities of the bispecific diabody with the parental scFvs suggests that all the non-covalent heterodimers (diabody) are correctly formed by specific V<sub>H</sub>-V<sub>L</sub> interactions. The affinity of the diabody for CD19-positive target B cells was 10-fold higher than its affinity for CD3 positive effector T cells.

### Stability of bispecific diabody in human serum

One might expect that the bispecific diabody would be rather labile, as it was formed by non-covalent interactions of 2 hybrid scFv molecules. We therefore investigated the stability of the diabody and both parental anti-CD3 and anti-CD19 scFvs when stored at a fairly low concentration in human serum at 37°C for prolonged periods of time. A concentration of 20  $\mu$ g/ml was chosen to avoid the fluorescence plateau in FACScan analysis (Kipriyanov *et al.*, 1997b). The residual antigen binding activity was estimated by flow cytometry. We found that the anti-CD3 scFv, which retained 70% of activity after 5 days of incubation, appeared to be significantly more stable than anti-CD19 scFv, which lost 50% of its activity after 36 hr of incubation and 95% after 4 days (Fig. 5). The diabody had an intermediate stability. It was less stable than the anti-CD3 scFv but slightly more stable than the anti-CD19 scFv (Fig. 5). The loss of both CD3 and CD19 binding had similar time kinetics, indicating an interdependent denaturation of the 2 binding domains.

### Induction of specific cytotoxicity by diabody

The ability of the bispecific diabody to induce tumor cell lysis by redirecting T cell-mediated cytotoxicity was investigated using PBMC from healthy donors as effector cells. After stimulating with soluble MAb OKT3 and IL-2 for 4 days, the cells were washed to remove remaining antibody and cultured in medium alone overnight. Human B cell lines Raji and Namalwa expressing CD19 were used as target cells. The effect of the bispecific diabody was measured using a standard [<sup>51</sup>Cr]-release assay with increasing



**FIGURE 2** – Analyses of purified scFv anti-CD3, scFv anti-CD19 and bispecific diabody. (a) 12% SDS-PAGE under reducing conditions. The gel was stained with Coomassie brilliant blue. (b) Analytical gel filtration on a Superdex 75 column. The elution buffer was PBS, pH 7.0. Sample volume and flow rate were 200  $\mu$ l and 0.5 ml/min, respectively. Molecular masses were calibrated with BSA (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa).

effector to target cell ratios. A mixture of both parental scFv fragments (anti-CD3 and anti-CD19) served as a negative control for determining the background lysis. Although the observed background lysis was different in different experiments, the diabody appeared to be quite potent in retargeting activated PBMC to lyse both target cell lines in a concentration-dependent manner (Fig. 6).

The CD3 × CD19 diabody was compared with a BsAb of the same specificity isolated from a hybrid-hybridoma OKT3 × HD37 (Csóka *et al.*, 1996). Bispecific diabody proved to be 10-fold more potent over the BsAb on a weight basis (3-fold on a molar basis) (Fig. 6a). Moreover, the increased cytotoxicity of the diabody was independent of the effector:target ratio (Fig. 6).

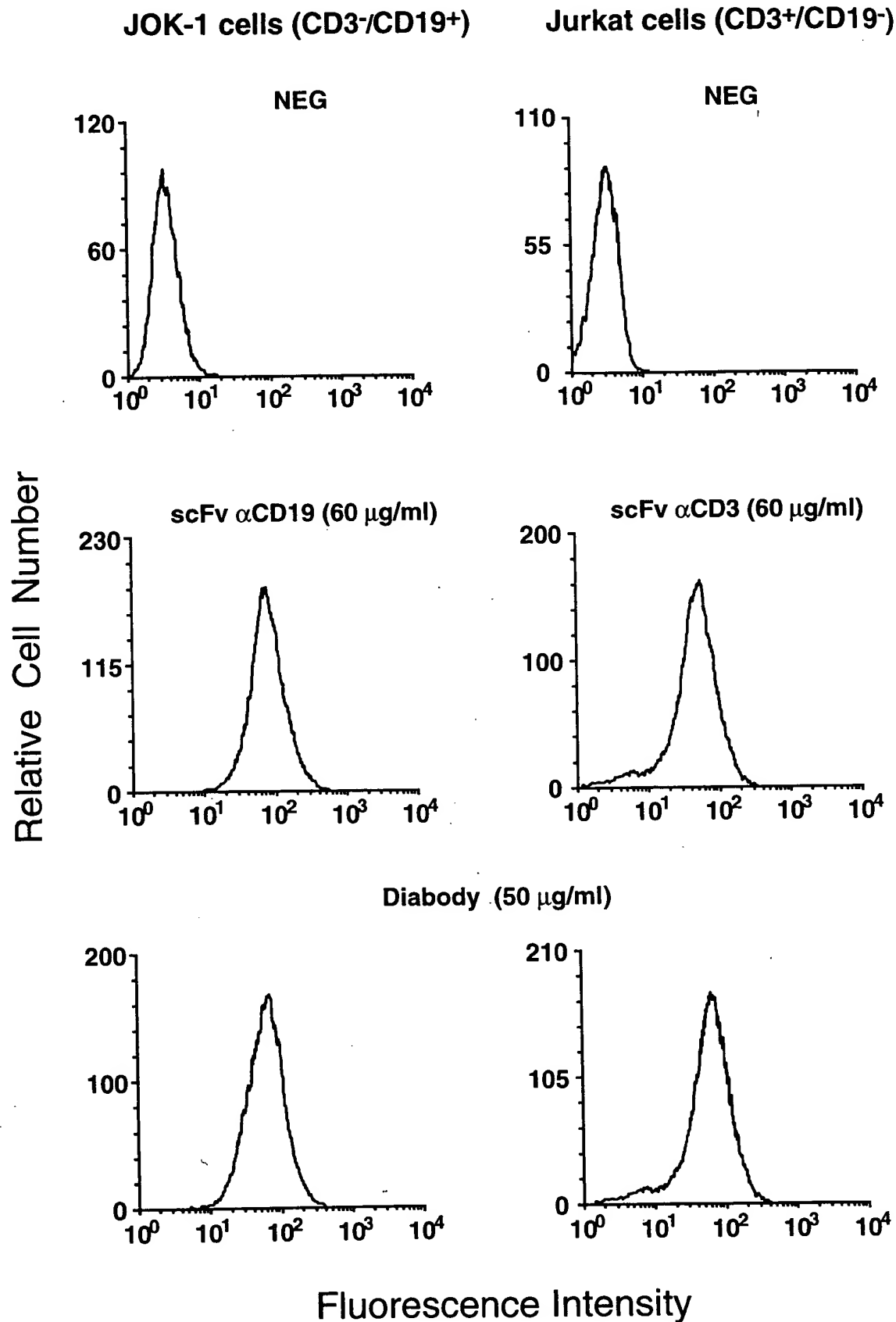
#### DISCUSSION

The 95 kDa CD19 antigen represents the broadest lineage-specific marker expressed on human B cells: it is present on the surface of virtually all B lymphocytes, including early B progenitor cells. CD19 is lost during the terminal stages of B cell differentiation but is exposed on the vast majority of B cell tumors (Uckun and Ledbetter, 1988). We therefore chose this antigen as the target molecule for BsAb-mediated cytotoxicity and constructed an anti-human CD19 scFv (Kipriyanov *et al.*, 1996).

Although different BsAb cross-linking NK cells (De Palazzo *et al.*, 1992) or activated neutrophils (Michon *et al.*, 1995) to tumor cells have been described, the most effective approach for tumor

rejection appears to be targeting via the CD3 molecule on cytotoxic T cells. The feasibility and effectiveness of this immunotherapeutic concept has been studied extensively in pre-clinical models, as well as in phase I clinical trials (Bolhuis *et al.*, 1996).

For the generation of a bispecific antibody suitable for therapy of human B cell malignancies, we aimed to construct a small recombinant molecule with dual specificity for both the human B cell surface antigen CD19 and the signal-transducing CD3 $\epsilon$  chain of the human TCR/CD3 complex. Although numerous anti-human CD3 MAbs have been used to study the T cell activation (Schwinzer *et al.*, 1992) and to create BsAb either by chemical conjugation (Anderson *et al.*, 1992; Nitta *et al.*, 1990) or by the quadroma technique (Bohlen *et al.*, 1993; Jacobs *et al.*, 1997), only 2 different anti-human CD3 antibody fragments have been used to date for the creation of recombinant BsAb. Variable domains derived from the hybridoma TR66 were used for constructing CD4-FvCD3 bispecific single chain molecules Janusins (Trautnecker *et al.*, 1991) and later for the creation of (scFv)<sub>2</sub> specific for both the epithelial 17-1A antigen and human CD3 (Mack *et al.*, 1995). In the second case, humanized V domains originally derived from the murine hybridoma UCHT1 were used for constructing anti-CD3/anti-p185<sup>HER2</sup> F(ab')<sub>2</sub> (Shalaby *et al.*, 1992) and a diabody (Zhu *et al.*, 1996). For constructing the BsAb described here, we used an scFv gene derived from the well-known hybridoma OKT3, which had been modified to improve its stability and folding in bacteria (Kipriyanov *et al.*, 1997b).



**FIGURE 3** – Flow cytometric analysis of scFv and diabody binding to CD19<sup>+</sup>/CD3<sup>-</sup> JOK-1 cells and CD3<sup>+</sup>/CD19<sup>-</sup> Jurkat cells. As a negative control, the binding of an irrelevant scFv was used.



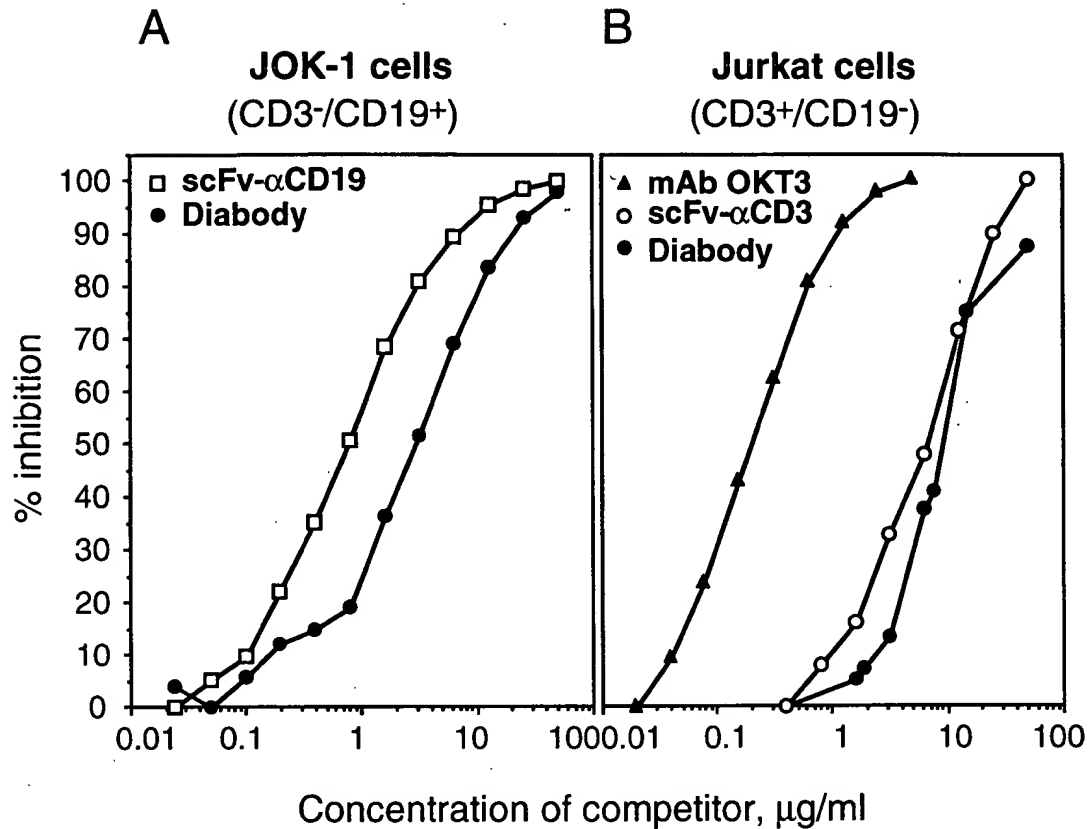


FIGURE 4 – Analyses of apparent affinities by flow cytometry. Inhibition of binding of FITC-labeled MAb HD37 to JOK-1 cells (a) and inhibition of binding of FITC-labeled MAb OKT3 to Jurkat cells (b) in the presence of MAb OKT3, scFv-αCD19, scFv-αCD3 and bispecific diabody are shown.

TABLE I – AFFINITIES OF MAb AND ANTIBODY FRAGMENTS

Antibody	IC <sub>50</sub> (nM)	K <sub>d</sub> (M <sup>-1</sup> ) <sup>1</sup>	K <sub>a</sub> (M <sup>-1</sup> ) <sup>2</sup>
JOK-1 cells			
MAb HD37	n.d. <sup>3</sup>	n.d.	2.5 × 10 <sup>9</sup>
scFv-αCD19	24.0	9.7 × 10 <sup>8</sup>	2.9 × 10 <sup>9</sup>
Diabody	48.8	4.8 × 10 <sup>8</sup>	n.d.
Jurkat cells			
MAb OKT3	1.3	7.7 × 10 <sup>8</sup>	n.d.
scFv-αCD3	222.2	4.5 × 10 <sup>7</sup>	n.d.
Diabody	156.0	6.4 × 10 <sup>7</sup>	n.d.

<sup>1</sup>Affinities determined from inhibition experiments. <sup>2</sup>Affinities determined from Scatchard plots. <sup>3</sup>Not determined.

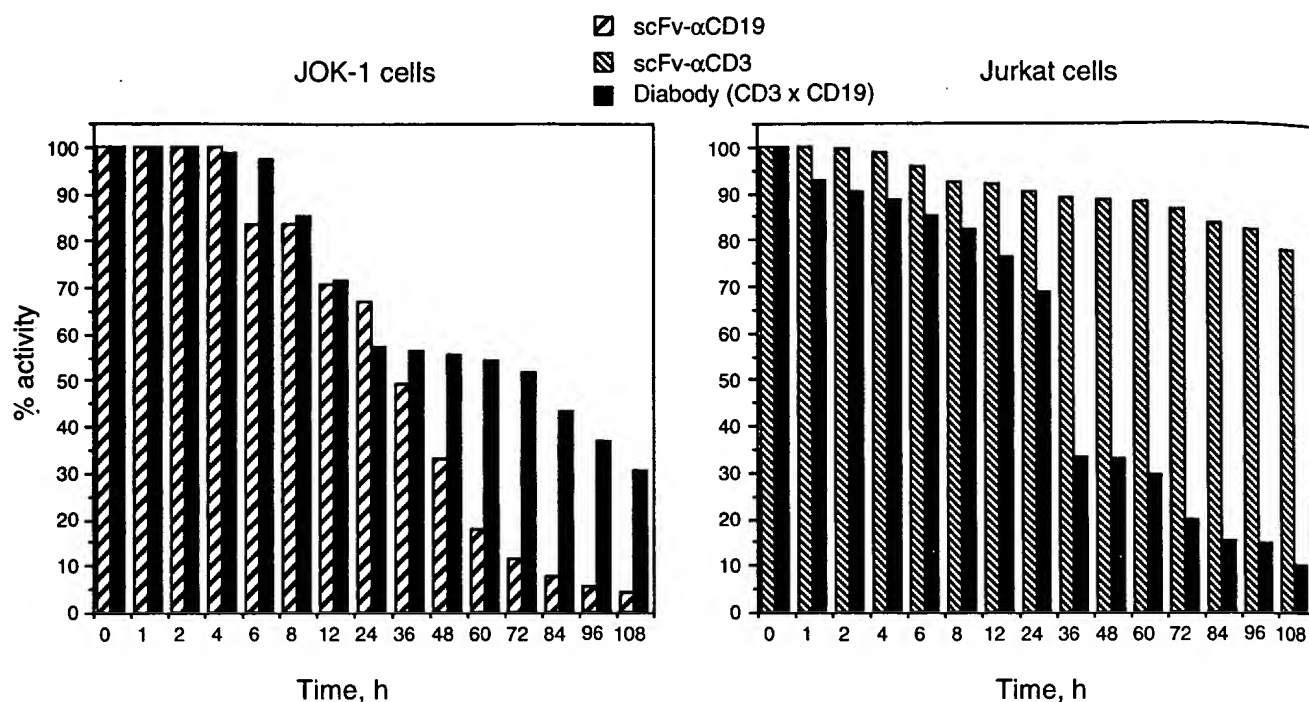
Initial attempts to produce bispecific CD3 × CD19 (scFv)<sub>2</sub> molecules, as recommended by Gruber *et al.* (1994), were unsatisfactory because of the small yields and low anti-CD3 activity (data not shown). We therefore constructed a more rigid cross-over scFv dimer (diabody). This construct has been shown to have 2 antigen-binding sites at opposite ends of the molecule, separated by 65 Å, which is sufficient to span the distance between 2 cells (Holliger *et al.*, 1996; Perisic *et al.*, 1994). The CD3 × CD19 diabody was produced by bacteria in a soluble functional form and could be retrieved from periplasmic extracts and culture medium in one step with a yield comparable to those obtained for parental scFv fragments.

Structurally, the isolated diabody was a stable compact dimer with an apparent m.w. around 50 kDa. Theoretically, the co-secretion of two hybrid scFv fragments may give rise to 2 types of dimer: active heterodimers and likely inactive homodimers. Affinity measurements indicated that the diabody was mostly, if not

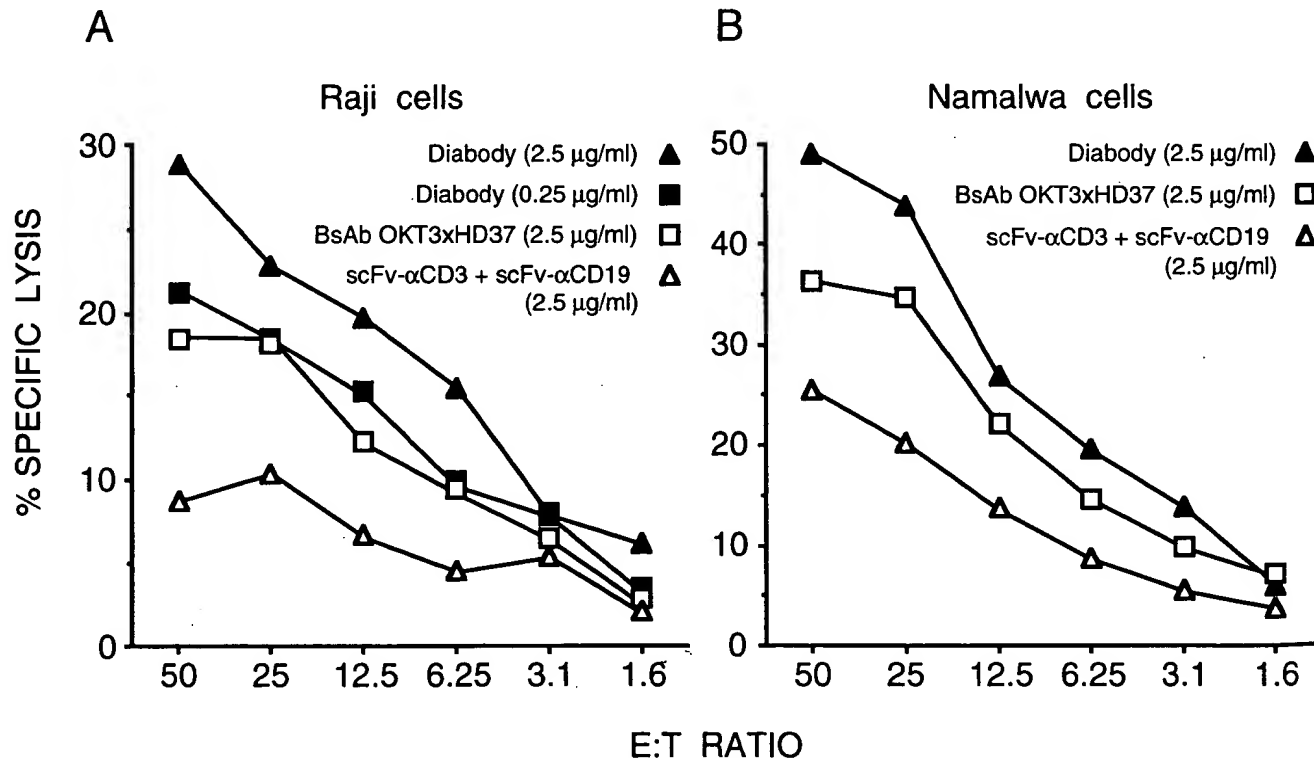
completely, in the active heterodimeric form. It bound to human CD3 with an affinity indistinguishable from that of the parental scFv-αCD3 and to human CD19 with an affinity about one half that of scFv-αCD19. This latter scFv has been shown to have a very high affinity in the subnanomolar range, similar to that of the parental MAb. Size-exclusion chromatography demonstrated that a significant part of this scFv was present in the form of non-covalent dimers and even higher oligomers, probably resulting in an enhanced affinity due to the increased avidity. In contrast, the CD19 binding domain of the diabody is only monomeric.

The higher affinity of the anti-CD19 moiety resulted in an almost 10-fold stronger binding of the diabody to the surface of target B cells than to the surface of T cells. Strong binding to a target tumor cell and weaker binding to an effector cell may have certain advantages for cancer therapy. For example, experiments *in vitro* demonstrated that the cytotoxic potential of recombinant BsAb does not depend on the affinity of its CD3-binding domain (Zhu and Carter, 1995). Furthermore, in a model of TCR serial triggering (Valitutti *et al.*, 1995), a high off-rate of the TCR is essential since it allows a single peptide-MHC complex to engage many TCRs in successive rounds of ligation, triggering and dissociation. Therefore, a higher affinity could result in less stimulation because the lower off-rate may prevent TCR reuse. A similar situation may also be present in the case of the surrogate antigenic stimulation of T cells through the anti-CD3 part of BsAb. For example, i.v. administration of a F(ab')<sub>2</sub> of OC/TR BsAb, which has a relatively high affinity for human CD3ε (10<sup>8</sup> M<sup>-1</sup>) (Jacobs *et al.*, 1997), induced a generalized *in vivo* activation with severe side effects (Tibben *et al.*, 1993), which most probably originated from target cell-independent direct activation of T cells and subsequent release of cytokines. Therefore, a recombinant BsAb that binds strongly to





**FIGURE 5** – Stability of scFv fragments and diabody in human serum at 37°C. The antibody fragments were incubated at 20 µg/ml in human serum at 37°C for the times shown. The CD19 and CD3 binding activity was assessed by flow cytometry using CD19<sup>+</sup>/CD3<sup>-</sup> JOK-1 and CD3<sup>+</sup>/CD19<sup>-</sup> Jurkat cells. Activity of the samples at point zero was taken as 100%.



**FIGURE 6** – Diabody-mediated lysis of CD19-positive B cells by activated human PBMC. The human CD19 expressing [<sup>51</sup>Cr]-labeled target cell lines Raji (a) and Namalwa (b) were co-incubated with effector CTLs at different effector/target ratios for 4 hr. Percent cytotoxicity was calculated based on [<sup>51</sup>Cr]-release in the presence of 2.5 µg/ml of parental scFvs, 0.25 µg/ml of diabody, 2.5 µg/ml of diabody or 2.5 µg/ml of BsAb OKT3 × HD37.

target tumor cells and significantly more weakly to the signal-transducing CD3 $\epsilon$  chain of the TCR/CD3 complex on effector cells may prove to have the most desirable properties.

An analysis of the stability of antibody fragments in human serum demonstrated a significant difference between scFv- $\alpha$ CD19 and scFv- $\alpha$ CD3. The present data confirm our previous observation that scFv derived from the hybridoma OKT3 and containing a Cys/Ser substitution in CDR-H3 has an enhanced stability (Kipriyanov *et al.*, 1997b). A comparison of the stabilities of the diabody and the parental scFvs, performed here suggests a stabilizing effect of the anti-CD3 moiety of the diabody on the CD19-specific moiety and also an interdependent denaturation of the 2 binding domains.

The bispecific diabody was able to induce an efficient lysis of the target cells by human CTLs. A comparison with a hybrid-hybridoma BsAb of the same dual specificity (Csóka *et al.*, 1996) indicated that the diabody was even more potent in recruiting activated T cells for cytotoxicity of the target cells. The increased

efficiency of the smaller diabody may be due to the closer proximity of the target cell and T cells connected by the diabody (Holliger *et al.*, 1996; Perisic *et al.*, 1994).

The prohibitive cost of preparing bispecific antibodies with the quadroma technology has made it difficult to start clinical trials. Recombinant antibodies, on the other hand, can be produced cheaply in large quantities using high cell density fermentation of bacterial cultures (Horn *et al.*, 1996; Zhu *et al.*, 1996). Furthermore, these smaller antibodies are less immunogenic due to the absence of constant domains. Consequently, we are now concentrating on the production of larger quantities of the diabody for testing in phase I clinical trials.

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#### REFERENCES

- ADAIR, J.R., ATHWAL, D.S., BODMER, M., BRIGHT, S.M., COLLINS, A., PULITO, V.L., RAO, P.E., REEDMAN, R., RÖTHERMEL, A.L., XU, D., ZIVIN, R.A. and JOLLIFFE, L.K., Humanization of the murine anti-human CD3 monoclonal antibody OKT3. *Hum. Antibodies Hybridomas*, **5**, 41–47 (1994).
- ANDERSON, P.M., CRIST, W., HASZ, D., CARROLL, A.J., MYERS, D.E. and UCKUN, F.M., G19.4( $\alpha$ CD3) × B43( $\alpha$ CD19) monoclonal antibody hetero-conjugate triggers CD19 antigen-specific lysis of t(4;11) acute lymphoblastic leukemia cells by activated CD3 antigen-positive cytotoxic T cells. *Blood*, **80**, 2826–2834 (1992).
- ATWELL, J.L., PEARCE, L.A., LAH, M., GRUEN, L.C., KORTT, A. and HUDSON, P., Design and expression of a stable bispecific scFv dimer with affinity for both glycoprotein and N9 neurominidase. *Mol. Immunol.*, **33**, 1301–1312 (1996).
- BIRD, R.E., HARDMAN, K.D., JACOBSON, J.W., JOHNSON, S., KAUFMAN, B.M., LEE, S.-M., LEE, T., POPE, S.H., RIORDAN, G.S. and WHITLOW, M., Single-chain antigen-binding proteins. *Science*, **242**, 423–426 (1988).
- BOHLEN, H., HOPFF, T., MANZKE, O., ENGERT, A., KUBE, D., WICKRAMANAYAKE, P.D., DIEHL, V. and TESCH, H., Lysis of malignant B cells from patients with B-chronic lymphocytic leukemia by autologous T cells activated with CD3 × CD19 bispecific antibodies in combination with bivalent CD28 antibodies. *Blood*, **82**, 1803–1812 (1993).
- BOLHUIS, R.L.H., HOOGENBOOM, H.R. and GRATAMA, J.W., Targeting of peripheral blood T lymphocytes. *Springer Semin. Immunopathol.*, **18**, 211–226 (1996).
- BRADFORD, M.M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254 (1976).
- BREITLING, F., DÜBEL, S., SEEHAUS, T., KLEWINGHAUS, I. and LITTLE, M., A surface expression vector for antibody screening. *Gene*, **104**, 147–153 (1991).
- BRENNAN, M., DAVIDSON, P.F. and PAULUS, H., Preparation of bispecific antibodies by chemical recombination of monoclonal immunoglobulin G1 fragments. *Science*, **229**, 81–83 (1985).
- CANEVARI, S. and 17 OTHERS, Regression of advanced ovarian carcinoma by intraperitoneal treatment with autologous T lymphocytes retargeted by a bispecific monoclonal antibody. *J. nat. Cancer Inst.*, **87**, 1463–1469 (1995).
- CARTER, P., RIDGWAY, J. and ZHU, Z., Toward the production of bispecific antibody fragments for clinical applications. *J. Hematother.*, **4**, 463–470 (1995).
- CSÓKA, M., STRAUSS, G., DEBATIN, K.-M. and MOLDENHAUER, G., Activation of T cell cytotoxicity against autologous common acute lymphoblastic leukemia (cALL) blasts by CD3 × CD19 bispecific antibody. *Leukemia*, **10**, 1765–1772 (1996).
- DE PALAZZO, I.G., HOLMES, M., GERCEL-TAYLOR, C. and WEINER, L.M., Antitumor effect of a bispecific antibody targeting CA19-9 antigen and CD16. *Cancer Res.*, **52**, 5713–5719 (1992).
- FANGER, M.W., MORGANELLI, P.M. and GUYRE, P.M., Bispecific antibodies. *Crit. Rev. Immunol.*, **12**, 101–124 (1992).
- GILL, S.C. and VON HIPPEL, P.H., Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.*, **182**, 319–326 (1989).
- GREENWOOD, F.C., HUNTER, W.M. and GLOVER, J.C., The preparation of <sup>131</sup>I-labeled human growth hormone of high specific radioactivity. *J. Biochem.*, **89**, 114–120 (1963).
- GROSSBARD, M.L., PRESS, O.W., APPELBAUM, F.R., BERNSTEIN, I.D. and NADLER, L.M., Monoclonal antibody-based therapies of leukemia and lymphoma. *Blood*, **80**, 863–878 (1992).
- GRUBER, M., SCHODIN, B.A., WILSON, E.R. and KRANZ, D.M., Efficient tumor cell lysis mediated by a bispecific single chain antibody expressed in *Escherichia coli*. *J. Immunol.*, **152**, 5368–5374 (1994).
- HOLLIGER, P., BRISSINCK, J., WILLIAMS, R.L., THIELEMANS, K. and WINTER, G., Specific killing of lymphoma cells by cytotoxic T-cells mediated by a bispecific diabody. *Protein Eng.*, **9**, 299–305 (1996).
- HOLLIGER, P., PROSPERO, T. and WINTER, G., “Diabodies”: small bivalent and bispecific antibody fragments. *Proc. nat. Acad. Sci. (Wash.)*, **90**, 6444–6448 (1993).
- HORN, U., STRITTMATTER, W., KREBBER, A., KNUPFER, U., KUJAU, M., WENDEROTH, R., MÜLLER, K., MATZKU, S., PLÜCKTHUN, A. and RIESENBERG, D., High volumetric yields of functional dimeric miniantibodies in *Escherichia coli*, using an optimized expression vector and high-cell-density fermentation under non-limited growth conditions. *Appl. Microbiol. Biotechnol.*, **46**, 524–532 (1996).
- HUSTON, J.S., MUDGETT HUNTER, M., TAI, M.S., MCCARTNEY, J., WARREN, F., HABER, E. and OPPERMAN, H., Protein engineering of single-chain Fv analogs and fusion proteins. *Methods Enzymol.*, **203**, 46–88 (1991).
- JACOBS, N., MAZZONI, A., MEZZANZANICA, D., NEGRI, D.R.M., VALOTA, O., COLNAGHI, M.I., MOUTSCHEN, M.P., BONIVER, J. and CANEVARI, S., Efficiency of T cell triggering by anti-CD3 monoclonal antibodies (mAb) with potential usefulness in bispecific mAb generation. *Cancer Immunol. Immunother.*, **44**, 257–264 (1997).
- KIPRIYANOV, S.M., DÜBEL, S., BREITLING, F., KONTERMANN, R.E. and LITTLE, M., Recombinant single-chain Fv fragments carrying C-terminal cysteine residues: production of bivalent and biotinylated miniantibodies. *Mol. Immunol.*, **31**, 1047–1158 (1994).
- KIPRIYANOV, S.M., KUPRIYANOVA, O.A., LITTLE, M. and MOLDENHAUER, G., Rapid detection of recombinant antibody fragments directed against cell-surface antigens by flow cytometry. *J. Immunol. Methods*, **196**, 51–62 (1996).
- KIPRIYANOV, S.M., MOLDENHAUER, G. and LITTLE, M., High level production of soluble single chain antibodies in small-scale *Escherichia coli* cultures. *J. Immunol. Methods*, **200**, 69–77 (1997a).
- KIPRIYANOV, S.M., MOLDENHAUER, G., MARTIN, A.C., KUPRIYANOVA, O.A. and LITTLE, M., Two amino acid mutations in an anti-human CD3 single chain Fv antibody fragment that affect the yield on bacterial secretion but not the affinity. *Protein Eng.*, **10**, 445–453 (1997b).
- KOSTELNY, S.A., COLE, M.S. and TSO, J.Y., Formation of a bispecific antibody by the use of leucine zippers. *J. Immunol.*, **148**, 1547–1553 (1992).
- KUNG, P.C., GOLSTEIN, G., REINHERZ, E.L. and SCHLOSSMAN, S.F., Monoclonal antibodies defining distinctive human T cell surface antigens. *Science*, **206**, 347–349 (1979).
- LAEMMLI, U.K., Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature (Lond.)*, **227**, 680–685 (1970).

- MACK, M., RIETHMÜLLER, G. and KUFER, P., A small bispecific antibody construct expressed as a functional single-chain molecule with high tumor cell cytotoxicity. *Proc. nat. Acad. Sci. (Wash.)*, **92**, 7021-7025 (1995).
- MICHON, J., MOUTEL, S., BARBET, J., ROMET-LEMMONNE, J.-L., DEO, Y.D., FRIDMAN, W.H. and TEILLAUD, J.-L., *In vitro* killing of neuroblastoma cells by neutrophils derived from granulocyte colony stimulating factor treated cancer patients using an anti-disialoganglioside/anti-FcγRI bispecific antibody. *Blood*, **86**, 1124-1130 (1995).
- MILSTEIN, C. and CUELLO, A.C., Hybrid hybridomas and their use in immunohistochemistry. *Nature (Lond.)*, **305**, 537-540 (1983).
- NITTA, T., SATO, K., YAGITA, H., OKUMURA, K. and ISHI, S., Preliminary trial of specific targeting therapy against malignant glioma. *Lancet*, **335**, 368-376 (1990).
- PERISIC, O., WEBB, P.A., HOLLIGER, P., WINTER, G. and WILLIAMS, R.L., Crystal structure of a diabody, a bivalent antibody fragment. *Structure*, **2**, 1217-1226 (1994).
- PEZZUTTO, A., DÖRKEN, B., RABINOVITCH, P.S., LEDBETTER, J.A., MOLDENHAUER, G. and CLARK, E.A., CD19 monoclonal antibody HD37 inhibits anti-immunoglobulin-induced B cell activation and proliferation. *J. Immunol.*, **138**, 2793-2799 (1987).
- SANGER, F., NICKLEN, S. and COULSON, R., DNA sequencing with chain terminating inhibitors. *Proc. nat. Acad. Sci. (Wash.)*, **74**, 5463-5467 (1977).
- SCATCHARD, G., The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.*, **51**, 660-672 (1949).
- SCHODIN, B.A. and KRANZ, D.M., Binding affinity and inhibitory properties of a single-chain anti-T cell receptor antibody. *J. biol. Chem.*, **268**, 25722-25727 (1993).
- SCHWINZER, R., FRANKLIN, R.A., DOMENICO, J., RENZ, H. and GELFAND, E.W., Monoclonal antibodies directed to different epitopes in the CD3-TCR complex induce different states of competence in resting human T cells. *J. Immunol.*, **148**, 1322-1328 (1992).
- SHALABY, M.R., SHEPARD, H.M., PRESTA, L., RODRIGUES, M.L., BEVERLEY, P.C., FELDMANN, M. and CARTER, P., Development of humanized bispecific antibodies reactive with cytotoxic lymphocytes and tumor cells overexpressing the HER2 protooncogene. *J. exp. Med.*, **175**, 217-225 (1992).
- TIBBEN, J.G., BOERMAN, O.C., CLAESSENS, R.A.M.J., CORSTENS, F.H.M., VAN DEUREN, M., DE MULDER, P.H.M., VAN DER MEER, J.W.M., KEUSER, K.G.G. and MASSUGER, L.F.A.G., Cytokine release in an ovarian carcinoma patient following intravenous administration of bispecific antibody OC/TR F(ab')<sub>2</sub>. *J. nat. Cancer Inst.*, **85**, 1003-1004 (1993).
- TRAUNECKER, A., LANZAVECCHIA, A. and KARJALAINEN, K., Bispecific single chain molecules (Janusins) target cytotoxic lymphocytes on HIV infected cells. *EMBO J.*, **10**, 3655-3659 (1991).
- UCKUN, F.M. and LEDBETTER, J.A., Immunobiologic differences between normal and leukemic human B-cell precursors. *Proc. nat. Acad. Sci. (Wash.)*, **85**, 8603-8607 (1988).
- VALITUTTI, S., MÜLLER, S., CELLA, M., PADOVAN, E. and LANZAVECCHIA, A., Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature (Lond.)*, **375**, 148-151 (1995).
- WINTER, G. and MILSTEIN, C., Man-made antibodies. *Nature (Lond.)*, **349**, 293-299 (1991).
- ZHU, Z. and CARTER, P., Identification of heavy chain residues in a humanized anti-CD3 antibody important for efficient antigen binding and T cell activation. *J. Immunol.*, **155**, 1903-1910 (1995).
- ZHU, Z., ZAPATA, G., SHALABY, R., SNEDECOR, B., CHEN, H. and CARTER, P., High level secretion of a humanized bispecific diabody from *Escherichia coli*. *Bio/Technology*, **14**, 192-196 (1996).

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